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The role of immune-mediated apparent competition in genetically diverse malaria infections

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Abstract

Competitive interactions between co-infecting genotypes of the same pathogen can impose selection on virulence, but the direction of this selection depends on the mechanisms behind the interactions. Here, we investigate how host immune responses contribute to competition between clones in mixed infections of the rodent malaria parasite *Plasmodium chabaudi.* We studied single and mixed infections of a virulent and an avirulent clone, and compared the extent of competition in immuno-deficient and immuno-competent mice ("nude" mice and T-cell reconstituted "nude" mice, respectively). In immuno-competent mice, the avirulent clone suffered more from competition than did the virulent clone. The competitive suppression of the avirulent clone was alleviated in immuno-deficient mice. Moreover, the relative density of the avirulent clone in mixed infections was higher in immuno-deficient than in immuno-competent mice. We conclude that immune-mediated interactions contributed to competitive suppression of the avirulent clone, although other mechanisms, presumably competition for resources such as red blood cells, must also be important. Because only the avirulent clone suffered from immune-mediated competition, this mechanism should contribute to selection for increased virulence in mixed infections in this host-parasite system. So far as we are aware, this is the first experimental evidence of immune-mediated apparent competition in any host-parasite system.

Introduction

Infections of pathogenic micro-organisms are often genetically diverse, with hosts infected by more than one genotype of the same pathogen species (Thompson 2000). Interactions between co-infecting genotypes in such mixed infections have been identified as a potentially important factor in the evolution of medically relevant pathogen traits, including drug resistance and virulence (Read and Taylor 2001). Here, we are primarily concerned with how within-host interactions influence the evolution of virulence (the degree to which a pathogen reduces its host's fitness).

The research field of virulence evolution has a solid theoretical foundation [reviewed by Frank (1996); for recent development of the theory, see e.g., Day and Proulx 2004; Alizon and van Baalen 2005)]. Briefly, faster exploitation of hosts and higher within-host density is assumed to increase a pathogen's between-host transmission rate. However, higher within-host density should also lead to increased virulence, which will shorten the time span for transmission because of host death. As a result of these conflicting selection pressures, there should be an intermediate optimum for virulence that maximizes a pathogen's fitness (between-host transmission success). However, this scenario concerns genetically uniform infections. When hosts are infected by multiple genotypes of the same pathogen species, there may be an additional selection pressure on virulence as a result of interactions between co-infecting genotypes. For example, it is often assumed that co-infecting genotypes compete over limited host resources. Such competitive interactions have traditionally been expected to select for faster exploitation of hosts and thereby higher virulence, because a "prudent" genotype that exploits hosts relatively slowly should be out competed by more virulent genotypes (Frank 1996). However, other forms of within-host interactions may also occur and recently it has been realized that the direction of selection on virulence imposed by such interactions depends on their mechanistic details (e.g., Taylor et al. 1997; Chao et al. 2000;

Read and Taylor 2000; Brown et al. 2002; West and Buckling 2003). There are several different types of potential interactions, including resource competition, interference competition and immune-mediated interactions. Here, we report an experimental analysis of the latter.

Interactions between two (or more) co-infecting pathogen genotypes mediated by host immune responses are analogous to interactions between two prey species mediated by a shared predator species. Such indirect interactions have received much attention in community ecology and are considered to play an important role in structuring ecological assemblages (Holt and Lawton 1994; Menge 1995). Interestingly, indirect effects mediated by a shared predator can have both negative and positive effects on the prey species depending on the behavioural and numerical response of the predator to a change in abundance of its prey. If an increased abundance of one prey increases the abundance of the predator (a numerical response), this can have a negative effect on the other prey, an effect termed "apparent competition" by Holt (1977). However, if an increased abundance of one prey leads the predator to divert its attention to this prey (a behavioural response), this can have a positive effect on the other prey, that is, "apparent commensalism" (Abrams and Matsuda 1996). The relative importance of numerical and behavioural responses will determine whether the net effect is a positive or negative interaction between prey species (Abrams and Matsuda 1996).

Applying this community ecology theory to immune-mediated interactions between co-infecting pathogen genotypes yields the following two scenarios for selection on virulence in mixed infections: First, if the immune response is genotype-transcending and the strength of the response increases with the density of parasites, so that a genotype that finds itself in a mixed infection encounters a stronger immune response than it would have induced on its own, this could result in immune-mediated apparent competition. Given that a more virulent

genotype induces a stronger response (because it has higher density), it seems likely that immune-mediated competition will be asymmetrical, such that an avirulent genotype suffers more from the presence of a virulent genotype than vice versa. If so, this kind of apparent competition should reduce the relative fitness of the avirulent genotype in mixed infections and thereby contribute to selection for increased virulence.

Second, if the immune response is genotype-specific, and the immune system focuses primarily on the most virulent genotype (because it is initially most abundant), it is possible that a genetically diverse infection can allow a genotype with relatively low exploitation rate to partly elude the attention of the immune system (Taylor et al. 1997; Read and Taylor 2000; Almogy et al. 2002), especially since the immune system has a tendency to retain its focus on the antigenic variant that stimulated the response originally, a phenomenon known as "original antigenic sin" (Janeway and Travers 1996). This can result in an avirulent genotype doing better in genetically diverse infections than it does alone (immune-mediated facilitation, analogous to apparent commensalism between prey species). This kind of interaction should increase the relative fitness of the avirulent genotype, and therefore has the potential to cause selection for reduced virulence in genetically diverse infections.

Results from empirical studies of the relationship between the virulence of a genotype and its performance in genetically diverse infections are mixed. In some cases, the most virulent genotype is also the most competitive, but several studies have found the opposite (reviewed in Read and Taylor 2000, 2001; see also Gower and Webster 2005; De Roode et al. 2005b). One explanation for these contrasting results is that the relative importance of different mechanisms of interaction differs between host-pathogen systems. To understand how genetically diverse infections affect the evolution of virulence we therefore need to elucidate the mechanistic basis of interactions between co-infecting genotypes. Here, we investigate how host immune defence contributes to interactions between clones in mixed

infections of the rodent malaria parasite *Plasmodium chabaudi. Plasmodium chabaudi* in laboratory mice is commonly used as a model system for human malaria (e.g. Langhorne et al. 2002). Understanding the role of the immune system in mediating interactions between co-infecting clones is particularly interesting in this model of an important human disease, because the extent of such interactions, and hence the direction and strength of selection on virulence in mixed infections, could be influenced by vaccination (Mackinnon and Read 2004a).

Previous experiments with *P. chabaudi* have shown that there is strong within-host competition between clones (Snounou et al. 1992; Taylor et al. 1997; De Roode et al. 2003; 2004b) and that relatively avirulent clones suffer most (De Roode et al. 2005b; A. Bell et al. unpubl. ms). How does host immune defence contribute to this pattern? *Plasmodium chabaudi* infections induce immune responses that are at least partially cross-reactive between clones (e.g. Jarra and Brown 1989; Buckling and Read 2001; Mackinnon and Read 2003) so that immune-mediated competition could be the proximate cause of the suppression of avirulent clones. However, there is some specificity of the response (Jarra and Brown 1989; Buckling and Read 2001; Martinelli et al. 2005). Thus, it is also possible that host immune defence alleviates competition caused by, for example, lack of resources. To elucidate whether host immune responses enhance or alleviate competition we performed an experiment with immuno-deficient mice. The immune response to *P. chabaudi* is initially cell-mediated, followed by the production of antibodies (Langhorne et al. 2002; Stevenson and Riley 2004). Both these responses are dependent on T helper cells. We therefore used nude mice (which are athymic and therefore lack T cells) to investigate if and how host immune defence contributes to interactions between co-infecting clones. Specifically, we compared the extent of competition between a virulent and avirulent *P. chabaudi* clone in immuno-deficient nude mice and immuno-competent mice (nude mice reconstituted with T

cells). If the competition observed in previous studies is at least partly a result of apparent competition through T cell mediated immunity, we expect that competition should be reduced in nude mice. In contrast, if clone-specific immunity alleviates other forms of competition, we expect competition to be more intense in nude mice than in reconstituted mice.

Methods

Plasmodium chabaudi

Like other *Plasmodium* species, *P. chabaudi* replicates asexually in red blood cells (RBCs). Infected RBCs rupture synchronously, each releasing 6-8 parasites, which infect new RBCs (Carter and Diggs 1977). In *P. chabaudi,* this asexual replication cycle is repeated every 24 h. The density of asexual parasites reaches a peak 4-11 days post-infection (depending on the inoculation dose), followed by a rapid decrease over the next few days (Timms et al. 2001). This acute phase of the infection is followed by a chronic phase with recurrent lower peaks. A small proportion (ca. 1%) of the asexuals differentiate into gametocytes, the stage responsible for transmission to new hosts. Gametocytes are mainly produced towards the end of the acute phase of the infection (Buckling et al. 1997). Asexual parasitaemia is positively correlated with gametocytaemia, which in turn predicts transmission success (Taylor and Read 1998; Mackinnon and Read 2004b).

We used two clones of *P. chabaudi,* denoted AS and AJ. Both clones were originally isolated from thicket rats (*Thamnomys rutilans*) in the Central African Republic (Beale et al. 1978). The clones are maintained as frozen stabilates. We use subscript codes to identify their position in the clonal history; the codes of the clones used here are AS_{11930} (derived from AS by selection for pyrimethamine and subsequently passaged several times through mice for maintenance purposes) and AJ_{4777}. AS has lower peak parasitaemia and is less virulent (causing less anaemia and weight loss in mice) than AJ (De Roode et al. 2004b).

Mice

We used female BALB/c-*nu/nu* mice ("nude mice") (Harlan, UK). *nu* is a recessive mutation that blocks the development of the thymus so nude mice have no mature T cells, whereas heterozygotes (*nu/+*) have a normal immune system (Pantelouris 1968). The nude mutation also has some pleiotropic effects. Most importantly, nudes are hairless and smaller. These pleiotropic effects could potentially affect resistance to infections. Consequently, it would be difficult to ascertain that a difference in within-host interactions between *nu/+* and *nu/nu* mice was due to the presence/absence of T cell dependent immunity. As controls, we therefore used *nu/nu* mice reconstituted with T cells, rather than *nu/+* mice. Reconstituted nude mice are nude mice that have received T cells from *nu/+* mice (see further below). Thus, nude mice and reconstituted nude mice differ only with respect to whether they have T cells or not.

Mice were kept in individually ventilated cages in a 12L:12D cycle. They were fed on 41B maintenance diet (Harlan, UK) and the drinking water was supplemented with 0.05% para-amino benzoic azid to enhance parasite growth (Jacobs 1964).

Reconstitution of nude mice by adoptive transfer of lymphocytes.

Pooled lymphocytes purified from spleen and lymph nodes of *nu/+* mice were depleted of B cells using CD19 magnetic beads (AUTOMACS® Miltenyi Biotech) according to the manufacturer's protocol. $15x10^6$ B cell depleted lymphocytes were then transferred intravenously into nude recipients when 10-12 weeks old.

Set-up and sampling

Mice of each phenotype were infected with 10^5 AS, 10^5 AJ or 10^5 AS+10^5 AJ parasites. We used the same dose of each clone in single and mixed infections (rather than the same total

dose in single and mixed infections) because the aim of the study was to compare the performance of a clone when it is on its own with when it is in a mixed infection. Thus, type of infection (single or mixed) and total infective dose are confounded. However, a twofold difference in infective dose has negligible effects on the population dynamics of the parasite (even a tenfold difference is barely detectable; Timms et al. 2001).

Among nude mice there were seven mice in each treatment group and among reconstituted mice there were six in each treatment. Inoculations were done contemporaneously, as described in De Roode et al. (2004b). Mice were 12-14 weeks old at infection.

We measured red blood cell density (using flow cytometry; Beckman Coulter) and took blood samples from the tail for PCR analysis (see below) and thin blood smears on days 0, 2, 4, and then daily up to day 18 post-inoculation, when the experiment was terminated. Blood smears were fixed with methanol and stained with Giemsa.

We quantified parasites in three ways: (i) Parasitaemia (the proportion of infected RBC) was determined by counting the number of infected cells per 500 red blood cells in at least four microscopic fields under 1000× magnification. Immunological and parasitological studies of rodent malaria have generally used this measure of infection intensity to estimate host resistance (i.e., the ability to control the intensity of the infection). To allow for comparison with previous studies, we therefore used parasitaemia as dependent variable when testing if the mouse phenotypes in our experiment differed in resistance. (ii) Parasite density (no. parasites/μl blood) was determined by clone-specific real-time quantitative PCR (qPCR) assays (see below), which allow us to estimate the density of each of the morphologically indistinguishable clones in mixed infections. We used this measure of infection intensity when investigating how the experimental treatment affected the performance of the different clones because it is a measure of the absolute number of parasites in a mouse, rather than

parasitaemia, which is a ratio of parasites to red blood cells. (iii) Parasite density was also determined by multiplying parasitaemia and RBC density values. Because different clones cannot be distinguished morphologically, this method cannot be used to estimate the density of each clone in mixed infections. However, we used estimates of parasite density in single infections obtained by this method to calibrate the qPCR assays (see below). 'Parasitaemia' and 'parasite density' give slightly different patterns of parasite dynamics (because parasite density depends on RBC density).

Quantitative PCR

Five µl of tail blood was taken from each mouse in the morning (when most parasites in the peripheral blood were in the ring or early trophozoites stages) and added to 100µl of citrate saline on ice. Samples were subsequently pelleted by centrifugation, the citrate saline removed and the blood stored at –80°C until required. DNA extraction was performed using the BloodPrep® kit (Applied Biosystems) on the ABI Prism® 6100 Nucleic Acid PrepStation according to manufacturer's instructions. DNA was eluted in a total volume of 200µl and stored at -80^0C until quantification. Clone-specific PCR primers and a common minor grove-binder (MGB) probe, targeting the *Plasmodium chabaudi ama1* gene, were designed using Primer Express® (Applied Biosystems) software. The amplicon length is 127 and 129 bp for clones AJ and AS, respectively. Clones AS and AJ were quantified in separate assays, using the appropriate clone-specific primers. 2µl of DNA was included in a 25µl volume PCR reaction with the following components: 1.5µl each of the forward (AS: 5' GGA AAA GGT ATA ACT ATT CAA AAT TCT AAG GT 3'; AJ: 5' GGA AAA GGT ATA ACT AAT CAA AAA TCT ACT AAA 3') and reverse primer (AS: 5' AAT TGT TAT AGG AGA AAT GTT TAC ATC TGT TTG 3'; AJ: 5' GTG TTA TAG GAG AAA TGT GTA CAT CTG TTT T 3'), both at a final concentration of 300nM; 12.5µl of TaqMan® Universal PCR Master Mix

(hot start); 1µl of MGB probe (5' 6-FAM-ATC CTC CTT CTC TTA CTT TC-MGB 3') at a final concentration of 200nM and 6.5µl of sterile water. Amplification was performed on an ABI Prism® 7000 real-time thermal cycler with an initial denaturation of 95ºC for 10min followed by 45 cycles of denaturation at 95ºC for 15s and annealing/extension at 60ºC for 1min. Absolute quantification of experimental samples was performed by comparing threshold cycle numbers against a standard curve covering 6 orders of magnitude. Standard samples were added in triplicate. We calibrated the standard curve by regressing the values obtained from the qPCR against the densities obtained from smear counts (i.e. proportion infected red blood cells × red blood cell density) for all single infections over day 5-12. Densities obtained from qPCR and smear counts were highly correlated (AS, r=0.93; AJ, r=0.94). Each qPCR run included DNA samples of the mouse host and the non-target clone as negative controls. The real-time qPCR assays used here do not discriminate between asexual parasites and gametocytes. Consequently parasite numbers obtained also include gametocytes (when present), although gametocytes are always two to three orders of magnitude less numerous than asexual parasites (e.g. Buckling et al. 1997; Buckling and Read 2001; Mackinnon and Read 2003) and hence contribute minimally to overall quantifications.

To compare the repeatability (Lessells and Boag 1987) of parasite densities obtained by qPCR and from smear counts (% infected RBCs × no RBC/µl), we performed a separate experiment where 10 mice were infected with AJ or AS. On days 5, 7, 10, 12, and 14 post-inoculation we took two blood samples for qPCR analysis, two blood smears and two measures of RBC density from each mouse. The two blood samples for qPCR analysis were extracted on different plates and quantified in different qPCR runs. The repeatability of parasite densities obtained by qPCR was 0.98, compared to a repeatability of 0.92 obtained from smear counts. We conclude that the qPCR technique is at least as repeatable as conventional methods.

Statistical analyses

The main analyses to investigate if and how T cell dependent immune responses influenced parasite and red blood cell dynamics were performed as repeated measures analyses, using data from the part of the acute phase when the immune response was effective (i.e., when there was a significant difference in parasitaemia between nude and reconstituted mice). These analyses were performed with PROC MIXED in SAS 8.2 (SAS Institute 1999) using the REPEATED statement (subject=mouse) and Satterthwaite approximation of the denominator df. Of the three covariance structures proposed for repeated measures analysis by Littell et al. (1996), the autoregressive covariance structure "AR(1)" generally gave the best fit, as assessed by the Bayesian information criterion, and we therefore used this for all analyses. All other analyses were performed with PROC GLM using type 3 sums-of-squares. Parasite density values were log-transformed because we are interested in testing for a proportionate difference in competitive suppression between different treatments. Analyses of clonal proportions in mixed infections were performed with angularly transformed values.

Results

Mortality

Of the nude mice with mixed infections, 1 died (after sampling) on day 10, 3 on day 11, 1 on day 12, and the last 2 on day 13. During this period (day 0-13), 4 nude mice infected with AJ died on day 11 and 1 nude mouse infected with AS died on day 12. None of the reconstituted mice died during this period. To avoid having too many missing values, analyses were restricted to day 0-12 unless otherwise stated.

Parasitaemia of AS and AJ in different mouse phenotypes

To test whether nude and reconstituted mice differed in resistance to *P. chabaudi*, we compared their parasitaemias (% infected RBC) in single infections (fig 1). In mice infected with AS, nude mice had higher parasitaemia than reconstituted mice from two days before the peak and onwards [significant difference ($P<.05$), as assessed by one-way ANOVA, from day 7 post inoculation]. In mice infected with AJ, nude and reconstituted mice did not differ in peak parasiteamia, but reconstituted mice cleared parasites faster than nude mice (significant difference from day 10). We thus conclude that reconstituted mice were indeed more resistant than nude mice.

As in previous studies (e.g., De Roode et al. 2004b), AJ had higher peak parasitaemia than AS, although this difference was only found in reconstituted mice (reconstituted: $F=6.1$, df=1, 10, $P=.033$; nude: $F=0.05$, df=1, 12, $P=.83$).

Competition in immuno-competent mice

We first tested if there was competition within the reconstituted mice. To allow comparison with previous studies that used C57 and CBA mice (De Roode et al. 2004b; De Roode et al. 2005b), we used the same statistical approach, that is, for each clone we compared its total parasite density during the acute phase (obtained by summing the daily densities during day 0-12) in single and mixed infections. AS was suppressed by 68% and AJ by 26% (AS: $F=108.2$, df=1, 10, $P<.001$; AJ: $F=29.5$, df=1, 10, $P<.001$). Thus, as in previous studies, competition between AS and AJ was asymmetrical, such that the avirulent clone AS suffered most.

Immune-mediated interactions

To investigate how T cell dependent immunity contributed to the competition observed in reconstituted mice, we tested for an interaction between mouse phenotype and type of infection (single or mixed). If competition is at least partly immune-mediated, there should be a significant interaction between mouse phenotype and type of infection, such that competitive suppression (the difference in parasite density a clone achieves in single and mixed infections) is reduced in nude mice. Alternatively, if immune-mediated facilitation alleviates other forms of competition, there should be a significant interaction such that the suppression is stronger in nude mice than in reconstituted mice.

We first ran a repeated measures analysis with parasite densities on day 7 through 12 as dependent variables. The analysis was restricted to day 7 and onwards because immune-mediated competition is only likely to occur during the later stage of the infection when the immune response is effective, and day 7 was the first day when reconstituted mice had lower parasitaemia than nude mice (fig 1). We then tested if the total parasite density during the acute phase of the infection was affected by immune-mediated competition. We analysed each clone separately.

Parasite clone AS. The density of AS over time in the different types of infections and mouse phenotypes is shown in fig 2a. A repeated measures analysis of day 7-12 (tab 1) showed that there were significant main effects of mouse phenotype and type of infection (single or mixed), such that AS densities were higher in nude mice than in reconstituted mice, and higher in single than in mixed infections. There was also a significant 3-way interaction between phenotype, infection, and day, indicating that T cell dependent immunity indeed had an effect on the extent of competitive suppression of AS in mixed infections, but that this effect varied over time. To investigate this further, we divided the data set in two parts— day 7-9 and day 10-12— and repeated the analysis with each of these (tab 1). As in the analysis of

day 7-12, there were significant main effects of phenotype and infection during both day 7-9 and 10-12, such that AS densities were higher in nude mice than in reconstituted mice, and higher in single infections than in mixed infections. However, the phenotype-by-infection interaction was only significant during day 10-12. Inspection of figs 2a and c shows that during day 10-12, the suppression of AS in mixed infections was greater in reconstituted mice than in nude mice. Thus, there was immune-mediated competition.

Could the immune-mediated competition that occurred towards the end of the acute phase account for the suppression of total number of AS parasites present during the acute phase observed in reconstituted mice? A two-way ANOVA revealed main effects of mouse phenotype (F=7.1, df=1, 21, P=.014) and type of infection (F=353.6, df=1, 21, P<.001), but there was no evidence of a phenotype-by-infection interaction (F=1.6, df=1, 21, P=.22). (As detailed above, mice started to die on day 10, so the calculation of total density was restricted to day 0-11 to maintain statistical power. An analysis including only the mice that survived to day 12 gave the same result.) Thus, there was no evidence that the overall competitive suppression of AS was caused by immune-mediated competition.

Parasite clone AJ. The density of AJ over time in the different types of infections and mouse phenotypes is shown in fig 2d. In the repeated measures analysis of day 7-12 (tab 2), there were significant main effects of mouse phenotype and type of infection, such that AJ densities were higher in nude mice than reconstituted mice, and in single infections than in mixed infections, but neither the phenotype-by-infection nor the 3-way interaction between phenotype, infection and day were significant. Although the three-way interaction was not significant, for comparison with the analysis of AS we performed analyses with day 7-9 and 10-12 separately, but in neither case was the interaction between mouse phenotype and type of infection significant (P≥.3; fig 2ef). In the analysis of total AJ numbers, single infections contained more AJ parasites than did mixed infections (F=75.2, df=1, 21, P<.001), but the

difference between mouse phenotypes was not statistically significant (F=3.4, df=1, 21, P=.079). There was no phenotype-by-infection interaction (F=0.1, df=1, 21, P=.73). Thus, there was no evidence that the extent of suppression of AJ in mixed infections was T-cell dependent.

Relative densities of clones in mixed infections

To test if the two clones were differently affected by the presence/absence of T cell dependent immunity when in a mixed infection, we compared the relative densities of the clones in nude and reconstituted mice. The density of AS and AJ over time in mixed infections in the different mouse phenotypes is shown in fig 3a. The analysis was performed as a repeated measures analysis with proportion AS against mouse phenotype, day and their interaction. As previously, the analysis was restricted to the period when host immune defence was effective, that is, day 7-12. There was a significant effect of day (F=78.0, df=5, 44.2, P<.0001), with proportion AS decreasing over time, and a significant effect of mouse phenotype (F=5.9, df=1, 18.1, P=.026), with reconstituted mice having a lower proportion of AS than nude mice. However, there was also a day-by-phenotype interaction (F=3.0, df=5, 44.2, P=.021), indicating that this effect varied over time. To investigate this further, we again divided the data set in two parts—day 7-9 and 10-12—and repeated the analysis with each of these. During day 7-9, there was no difference in proportion AS between phenotypes (phenotype: F=1.4, df=1, 15.4, P=.26; day: F=20.6, df=2, 24.4, P<.0001; phenotype x day: F=1.7, df=1, 24.4, P=.20; fig 3b). During day 10-12, reconstituted mice had a lower proportion of AS than nude mice (phenotype: F=5.6, df=1, 11.4, P=.037; day: F=3.4, df=1, 17.8, P=.054; phenotype x day: F=3.9, df= 1, 17.8, P=.039; fig 3b). Thus, T cell dependent immunity reduced the relative density of AS in mixed infections, but this effect was only significant during day 10-12.

RBC density

Uninfected RBCs form an important resource for malaria parasites. To assess whether the potential for competition over this resource differed between mouse phenotypes, we compared the RBC density of nude and reconstituted mice with mixed infections (fig 4). The statistical analysis was performed in the same way as the analyses of parasite densities above, that is, as a repeated measures analysis of day 7-12 . There were significant effects of day (F=129.5, df=5, 47.2, P<.0001) and phenotype (F=7.4, df=1, 10.1, P=.022), with nude mice having lower RBC density than reconstituted mice. There was also a significant interaction between day and phenotype (F=4.5, df=5, 47.2, P=.002). We therefore repeated the analysis with day 7-9 and 10-12 separately. The difference in RBC density between phenotypes was significant during both these periods (day 7-9: F=5.5, df=1, 11.8, P=.038; day 10-12: F=12.4, df=1, 11.1, P=.0048), although the effect was apparently stronger during the latter period. Thus, although nude mice lost more RBC than reconstituted mice, AS suffered more from competition in reconstituted mice than in nude mice, implying that the immune response played a large part in causing this competition.

Discussion

We found that the competitive suppression of the avirulent clone AS was alleviated towards the end of the acute phase of the infection (day10-12 post infection) in immuno-deficient nude mice as compared to immuno-competent reconstituted mice. Thus, AS suffered from immune-mediated apparent competition. In contrast, there was no significant effect of mouse phenotype on the suppression of the virulent clone AJ. Apparent competition has previously been demonstrated in several different types of ecological assemblages—between prey species sharing a predator, between plants sharing a herbivore, and between hosts sharing a

parasite or parasitoid (reviewed by Chaneton and Bonsall 2000)—but the present study is to our knowledge the first to experimentally demonstrate apparent competition between co-infecting pathogen clones mediated by host immune responses. Apparent competition between prey, plant or host species is typically asymmetrical, such that only one of the victim species is affected by the presence of the other (Chaneton and Bonsall 2000), as we found too.

Relative importance of immune-mediated competition

The immune-mediated competitive suppression of AS occurred only towards the end of the acute phase of the infection. Because the parasite density during this period is low compared to the peak density, this immune-mediated competition did not have a measurable effect on the total number of parasites present during an infection. The competition that occurred around the peak of the acute phase (day 7-9), which accounts for most of the reduction in total parasite density, must instead have been caused by mechanisms other than T cell dependent immunity. Furthermore, even during day 10-12, there was strong competition also in the absence of T cell dependent immunity (Fig. 2). Thus, other mechanisms are clearly important also during this period. Which are these other mechanisms?

First, it could be T cell independent innate immune responses. Innate and adaptive immunity to malaria are intimately linked. For example, $CD4^+$ T cells produces IFN-γ which stimulates production of anti-parasite molecules such as nitric oxide by macrophages (Stevenson and Riley 2004). It is nonetheless possible that parts of the innate immune system could contribute to resistance in the absence of T cells. Further, a common finding is that mutant mice that lack specific components of the adaptive immune system compensate for their lack of adaptive immunity by a higher activity of innate immunity (e.g. Kaufmann and Ladel 1994). Thus, even though T cell dependent immunity is considered to be by far the most important component of defence against malaria (Langhorne et al. 2002; Stevenson and

Riley 2004), the redundancy of the immune system should make our estimate of the significance of immune-mediated competition conservative.

Second, competition could be caused by lack of resources, such as RBCs or glucose. The RBC density decreases dramatically during the infection, and lack of uninfected RBCs is known to affect parasite population growth in single infections (Yap and Stevenson 1994). It is therefore plausible that red cell availability plays an important role also in competition (Hellriegel 1992). The minimum RBC density was lower in nude mice than in reconstituted mice. Hence, the potential for competition over this resource was higher in nude mice. It is possible that increased competition for resources in nude mice partly obscured the release from immune-mediated competition. Again, this should make our estimate of the importance of immune-mediated competition conservative.

Third, an interesting possibility is that competition is due to direct interference between co-infecting genotypes, although such allelopathic mechanisms have so far only been described in bacteria and viruses (Hart and Cloyd 1990; Riley and Wertz 2002).

How general are our results?

Can we expect the extent and outcome of immune-mediated competition observed in the present study to be typical for *P. chabaudi?* One factor that can affect the extent of immune-mediated competition is the antigenic similarity of co-infecting clones. The more antigenically similar co-infecting clones are, the more immune-mediated competition should be expected. Experiments where mice were immunized with one clone and then challenged with mixtures of these clones showed that the responses to AJ and AS had a relatively high degree of cross-reactivity (K. Grech, B. Chan, A. F. Read, unpubl. data). It therefore seems unlikely that another clone combination would have shown markedly stronger immune-mediated competition. Another factor that can affect the extent of immune-mediated

competition is the timing of infections. In our experiment, infections were simultaneous, but in nature, infections will sometimes be sequential. Experiments with *P. chabaudi* have shown that competition is stronger when infections are sequential (De Roode et al. 2005a). This could be because the second clone encounters an environment with fewer resources (e.g. RBCs) or a stronger immune response. That immune-mediated competition is potentially important when infections are sequential is demonstrated by studies where mice have been immunized with one clone, cured and allowed to recover, and then inoculated with a heterologous clone. Such experiments have without exception found that immunity to one clone suppresses heterologous clones (e.g., Buckling and Read 2001; Mackinnon and Read 2003). Still, the relative importance of immune-mediated and resource competition when infections are sequential remains to be determined.

Is the extent of immune-mediated competition in this rodent malaria system representative for human malaria? *Plasmodium chabaudi* shares many features with the most virulent of the *Plasmodium* species infecting humans, *P. falciparum.* However, there is also an important difference that could affect competition; the peak parasite density is about an order of magnitude lower in *P. falciparum* than in *P. chabaudi* (Mackinnon and Read 2004b). It seems likely that a lower parasite density will reduce competition for resources, for example uninfected RBCs. If anything, the relative importance of immune-mediated competition could therefore be expected to be higher in *P. falciparum* than in *P. chabaudi* infections.

Evolutionary consequences of apparent competition

The evolutionary consequences of immune-mediated competition depend on how it affects the relative fitness of the co-infecting clones, which in turn depends on how it affects the production of transmission stages (gametocytes) and subsequent transmission to mosquitoes.

In the present study, we could only measure the density of asexual parasites, but previous studies have shown that the frequency of a clone among the asexual parasites in mixed infections predicts its transmission success (Taylor and Read 1998; De Roode et al. 2005b). We found that only the avirulent clone was affected by immune-mediated competition. Moreover, in mixed infections the avirulent clone obtained a lower share of the overall parasite density in immuno-competent as compared to immuno-deficient hosts. Even though these effects were quite weak and only occurred towards the end of the acute phase of the infection, they could still have a significant effect on the relative fitness of co-infecting clones, because gametocytes are generally mainly produced towards the end of the acute phase (e.g., Buckling et al. 1997). Taken together, this implies that the relative fitness of the avirulent clone in mixed infections was lower in immuno-competent than in immuno-deficient mice. By just using two clones to investigate how virulence influences sensitivity to immune-mediated competition we cannot formally establish a general relationship between these traits; there is a possibility that sensitivity to immune-mediated competition is determined by another unknown trait which is unrelated to virulence across a wider range of clones. However, it is difficult to see what kind of trait this could be which would not also affect virulence and we therefore tentatively conclude that the link between virulence and sensitivity found here represents a general pattern. If so, T cell dependent immunity should contribute to selection for increased virulence in mixed infections.

Large-scale vaccination campaigns against malaria could have the undesirable consequence of prompting evolution of parasites that are more virulent to unvaccinated hosts (Gandon et al. 2001; Mackinnon and Read 2004a). In several mathematical models, evolution of higher virulence occurs because vaccines that reduce parasite growth rate or toxicity also reduce the cost of virulence for the parasite (i.e., host death), which in turn causes between-host selection for more virulent parasites (Gandon et al. 2001; 2002; 2003). Vaccination could

also change the fitness function for the parasite by affecting the extent of within-host selection in mixed-genotype infections. For example, serial passage of malaria parasites increases their virulence, but parasites passaged through immunised hosts become virulent more rapidly (Mackinnon and Read 2004a). Mixed-genotype infections are common in human malaria, often exceeding 50 per cent of all infections (Babiker et al. 1999), so it is important to also assess how immune-imposed selection will affect within-host selection on virulence. The malaria vaccine candidates currently considered induce T cell dependent immunity (Good 2005), and the present study showed that T cell dependent immune responses contribute to competition. Hence, it seems likely that vaccination will increase the strength of competition. We are currently directly testing this possibility experimentally. For the moment we note that the result of the present study—that the relatively avirulent genotype suffered most from immune-mediated competition—could explain why experimental virulence evolution was more rapid in immunised animals (Mackinnon and Read 2004a). To the extent that the experimental results we report here generalize, we expect many types of vaccine to exacerbate immune-mediated competition, thus imposing selection for increased virulence.

However, a beneficial flip side of vaccine-enhanced immune-mediated competition is that it could retard the spread of drug resistance. Resistant mutants often compete with wild-type parasites in mixed infections when they first arise, and then with unrelated susceptible strains in mixed infections as they spread through a population. It seems highly likely that resistant strains will be less competitively successful (or they would have spread anyway), so that in-host competition in untreated hosts acts to slow the spread of drug resistance (Hastings and D'Alessandro 2000; De Roode et al. 2004a). If vaccination enhances immune-mediated competitive suppression, drug-resistant mutants will have an enhanced disadvantage in untreated patients and thus spread more slowly. The extent to which competitive suppression can be used to affect the spread of drug resistance has been little considered, but the

possibility that vaccination could slow resistance evolution, thus enhancing the useful life of chemotherapeutic agents, seems to us an idea worthy of theoretical and empirical consideration.

Finally, we note that when considering potential evolutionary consequences of competition between co-infecting pathogen genotypes, evolutionary ecologists have mainly focused on selection for competitiveness (and ensuing evolution of virulence). However, within-host competition can also lead to selection on other traits than competitiveness. Indeed, in other areas of evolutionary ecology, competition has received attention mainly as a potential factor selecting for phenotypic diversification (e.g., Schluter 2000). There is now strong empirical evidence that resource competition promotes phenotypic divergence (in traits affecting resource utilization)(Schluter 2000). It has also been proposed that apparent competition can promote divergence (in anti-predator traits), although empirical evidence for this process is as yet scarce (Abrams 2000). Like competition in other ecological assemblages, competition between co-infecting pathogen genotypes could also potentially select for phenotypic divergence. For example, resource competition could select for divergence in tissue tropism (Frank 2002), while immune-mediated apparent competition could select for divergence in antigenic profile (Gupta and Maiden 2001). One would expect that such divergence should reduce competition between co-infecting pathogens and thus reduce selection on competitiveness. Selection for competitiveness and phenotypic divergence therefore represent two alternative outcomes of within-host competition. Importantly, if within-host competition selects for divergence rather than competitiveness, it will not necessarily influence the evolution of pathogen virulence. Given that mixed-genotype infections of pathogens are common (Read and Taylor 2001), it would be of great biomedical interest to investigate what determines when within-host competition results in selection for phenotypic divergence or enhanced competitiveness.

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Table 1. Repeated measures analyses of the density of parasite clone AS in mixed and single infections in nude and reconstituted mice

	day 7-12				day 7-9				day 10-12			
Effect	Z	df	*F*	*P*	Z	df	*F*	*P*	Z	df	*F*	*P*
Mouse	8.8			<.0001	1.6			.10	8.59			<.0001
Phenotype		1, 25.7	13.2	.0012		1, 21.3	7.6	.012		1, 22.6	12.1	.0020
Infection		1, 25.7	293.5	<.0001		1, 21.3	286.6	<.0001		1, 22.6	209.0	<.0001
Day		5, 93.6	249.0	<.0001		2, 40.4	20.8	<.0001		2, 39.4	138.9	<.0001
Phen.*Inf.		1, 25.7	3.0	.096		1, 21.3	0.9	.36		1, 22.6	7.5	.012
Phen.*Day		5, 93.6	2.7	.026		2, 40.4	0.5	.62		2, 39.4	3.6	.036
Inf.*Day		5, 93.6	23.8	<.0001		2, 40.4	43.2	<.0001		2, 39.4	2.5	.095
P*I*D		5, 93.6	3.4	.0070		2, 40.4	0.6	.57		2, 39.4	0.9	.41

Note: The factors mouse phenotype (nude or reconstituted), type of infection (single or mixed) and day of infection were treated as fixed effects, while mouse individual was treated as a random effect.

Table 2. Repeated measures analysis of the density of parasite clone AJ in mixed and single infections in nude and reconstituted mice

		day 7-12		
Effect	Z	df	*F*	*P*
Mouse	4.2			<.0001
Phenotype		1, 34.2	14.2	.0006
Infection		1, 34.2	38.4	<.0001
Day		5, 86.3	163.5	<.0001
Phen.*Inf.		1, 34.2	1.3	.27
Phen.*Day		5, 86.3	7.9	<.0001
Inf.*Day		5, 86.3	2.8	.023
P*I*D		5, 86.3	1.6	.16

Note: The factors mouse phenotype (nude or reconstituted), type of infection (single or mixed) and day of infection were treated as fixed effects while mouse individual was treated as a random effect.

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Fig legends

Fig 1. Parasitaemia (% infected RBCs; mean±se) over time in nude and reconstituted mice. *A,* Mice infected with parasite clone AS. *B,* Mice infected with clone AJ.

Fig 2. Density of parasite clone AS and AJ in single and mixed infections in nude and reconstituted mice. *A,* Density (mean±se) of AS over time. *B,* Average density (LS means±se from repeated measures analysis) of AS on day 7-9. *C,* Average density of AS on day 10-12. *D,* Density of AJ over time. *E,* Average density of AJ on day 7-9. *F,* Average density of AJ on day 10-12.

Fig 3. Relative success of parasite clone AS and AJ in mixed infections in nude and reconstituted mice. *A,* Density of AS and AJ over time in mixed infections in nude and reconstituted mice. *B,* Average (LS means±se from repeated measures analysis) proportion of AS in mixed infections during day 7-9 and 10-12 in nude and reconstituted mice.

Fig 4. Red blood cell density (mean±se) over time in nude and reconstituted mice with mixed infections. Note that day 1-4 (when there is no change in RBC density) are omitted to enhance resolution.

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Fig 1 Råberg et al.

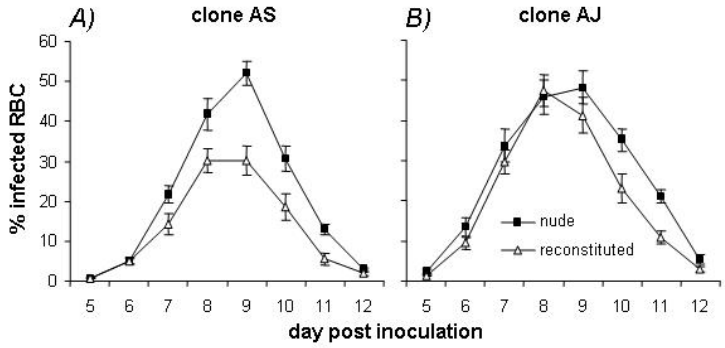

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Fig 2 Råberg et al.

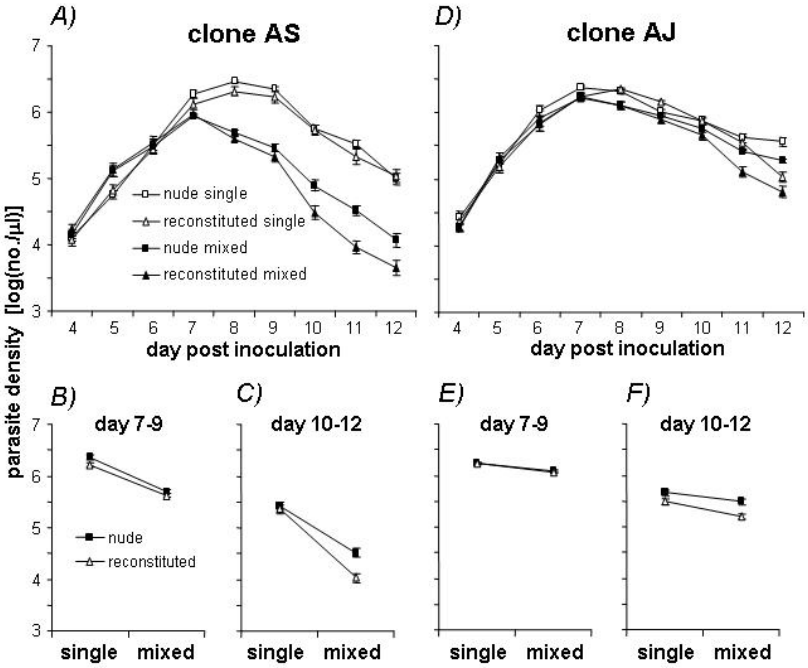

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Fig 3 Råberg et al.

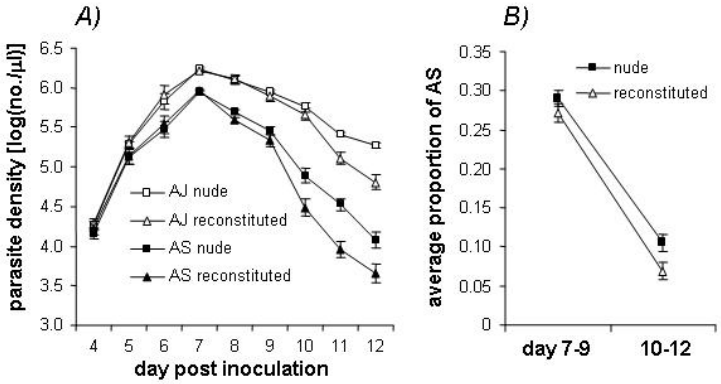

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Fig 4 Råberg et al.